Meat Flavor. 2. Procedures for the Separation of Water-Soluble Beef Aroma Precursors

SUMMARY—Water-soluble, low molecular weight beef aroma precursors have been separated by column chromatography on Bio-Gel P-2, Amberlite XAD-2 and DEAE-Sephadex. Separations on the basis of gel filtration, adsorption, and anion exchange resulted in a number of fractions that developed roast beef aroma on pyrolysis, but differed in composition. Either sugar phosphates or free sugars are involved in aroma development. Tyrosine, phenylalanine, taurine and glutamic acid may be removed without affecting aroma. A number of amino acids were present in the aorma producing fractions in trace amounts, thus the requirement for their presence may be questionable. Creatine, creatinine and the purine derivatives (inosinic acid, inosine, and hypoxanthine) may also be removed without affecting aroma development.

INTRODUCTION

PRECURSORS OF THE CHARACTERISTIC AROMA of meat are low molecular weight, water-soluble compounds that are easily extracted from muscle tissue. So many individual components are present, however, that it has not been possible to identify the compounds most directly associated with aroma development. Separations on ion exchange resins have given promising results (Batzer *et al.*, 1960, 1962; Wasserman *et al.*, 1965).

Since our previous study on the fractionation of flavor precursors on strong ion exchange resins (Wasserman *et al.*, 1965), we felt that the strongly active resins, as well as acid and alkali solutions needed to elute compounds from chromatographic columns, might induce alterations in the structure of some molecules, thus introducing artifacts into the study. To investigate milder separation techniques we turned toward gel filtration, which involves separation of molecules on the basis of molecular weight (Porath *et al.*, 1959; Gelotte, 1960). Small molecules which diffuse into the gel particles are retarded, while large molecules which are completely excluded from the gel phase will migrate through the column without retention in the interstitial fluid.

Gel filtration on crosslinked dextran, Sephadex G-25, has been mentioned previously in connection with fractionation of beef muscle components (Batzer *et al.*, 1960; Wasserman *et al.*, 1965). However, we employed a crosslinked polyacrylamide, Bio-Gel P-2, a comparatively new gel filtration material of smaller pore size than previously available, which suggested that separation of lower molecular weight compounds may be possible.

While our studies involving separation of sugars, amino acids and purine derivatives were in progress, three papers on the use of Bio-Gel P-2 appeared. Schwartz *et al.* (1966) reported on the desalting of peptides and amino acids. From their data possible separation of several amino acids can be inferred. Schwartz *et al.* (1965), as well as Uziel *et al.* (1965) reported on the use of P-2 gel for desalting and separating nucleic acid components on the basis of molecular weight.

A new adsorbent has recently become available for separating water-soluble organic compounds. The crosslinked polystyrene resin, Amberlite XAD-2, contains no functional groups; separations on this resin are based primarily on hydrophobicity. Molecules containing nonpolar groups or aromatic nuclei are adsorbed on the resin and are eluted at a slower rate than highly polar molecules. To our knowledge there are no publications dealing with the uses of Amberlite XAD-2. However, on the basis of information in the manufacturer's technical bulletin we felt this resin could give a series of useful separations of the meat components.

The weak anion exchange material, DEAE-Sephadex, is useful for selective removal of anions. It has been employed to separate basic peptides from the pituitary (Porath *et al.*, 1961), amino acids and peptides (Carnegie, 1961) as well as glycogen, sugars, sugar phosphates and adenine nucleotides from blood cells (Öckerman, 1963).

Results reported in this paper describe the use of Bio-Gel P-2, Amberlite XAD-2, and DEAE-Sephadex in the separation of water-soluble compounds in a continuing study on the isolation and characterization of meat aroma precursors.

EXPERIMENTAL

Preparation of beef diffusate

One kilogram of frozen beef muscle was partially thawed, cut into small pieces and homogenized with 4 L of cold deionized water for 2 min in a blendor. The homogenate was allowed to stand for 2 hr and the solids were then separated by centrifugation. The supernatant was dialyzed against deionized water for 72 hr with three changes of water of 3 L each, using Visking dialysis tubing, prepared by exhaustive washing with water to remove glycerin. The entire operation was carried out at 4°C. The combined diffusates were lyophilized, dissolved in deionized water to a final volume of 250 ml and stored at −18°C until used.

The dry weight of the diffusate (96 mg/ml) constitutes 2.4% by weight of the raw beef muscle.

Chromatography on Bio-Gel P-2

Bio-Gel P-2 (100–200 mesh), purchased from Bio-Rad Laboratories, was washed exhaustively with ethanol to remove ultraviolet absorbing impurities. The gel was hydrated with water and fines were removed by decantation.

A chromatographic column (2.4 × 75 cm), pretreated with 1% trimethylchlorosilane in carbon tetrachloride to reduce "wall effects," was packed with hydrated gel to a height of 68 cm. Beef diffusate (1.7 ml) was diluted to 5 ml with water and applied to the top of the gel bed. Components were eluted with water at a flow rate of 27 ml/hr and 6.9 ml fractions were collected.

Chromatography on DEAE-Sephadex

DEAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden) was washed and converted to the formate form as suggested by the manufacturer. The gel was equilibrated with 0.1*N* formic acid and packed to give a column 1.5 × 87 cm. Beef diffusate (1 ml), diluted with 1 ml 0.1*N* formic acid, was applied to the column and eluted with 0.1*N* formic acid at a flow rate of 0.5 ml/min. Four ml fractions were collected. After analysis, appropriate fractions were combined, lyophilized and redissolved in a minimum quantity of water.

Chromatography on Amberlite XAD-2

Crosslinked polystyrene polymer Amberlite XAD-2 (20–50 mesh, Rohm and Haas Co.) was washed extensively with methylene chlorine-methanol (1:3 v/v), methanol, and finally water to remove soluble impurities. The resin was suspended in water and poured into a chromatographic column to give a resin bed 2.9 × 34.5 cm. Beef diffusate (5 ml) was applied to the column and eluted with water at a flow rate of 1 ml/min while 4 ml fractions were collected. After analysis, appropriate fractions were combined, lyophilized and redissolved in a minimum quantity of water.

Analytical methods

Chromatographic column effluents were collected with a Beckman Model 132 Fraction Collector maintained at 5–10°C. The ultraviolet absorbance of the effluents was monitored at 248 mμ with a Gilford Model 2000 Multiple Sample Absorbance Recorder. Fractions were analyzed for total amino acids by the ninhydrin method of Cocking *et al.* (1954) and for carbohydrates with the anthrone method used by Toennies *et al.* (1964). Ultraviolet spectra were recorded with a Bausch and Lomb Spectronic 505 and absorbance in the visible region was measured with a Beckman Model B Spectrophotometer. Amino acid analyses were performed according to Spackman *et al.* (1958) using a Phoenix automatic amino acid analyzer.

The phosphorus analysis was carried out by a modified molybdenum blue spectrophotometric method (AOAC, 1965).

Thin-layer chromatography

Qualitative analytical determinations of fraction components were carried out by thin-layer chromatography on plates of Silica Gel G (Merck), Cellulose MN 300 (Macheray and Nagel), and DEAE-Cellulose (Serva), and Eastman Chromagram sheets (type K301R2).

The following solvent systems were used:

A. n-propanol-ammonia (70:30 v/v)
B. 88% phenol (Mallinckrodt)-water (100:10 v/v)
C. i-propanol-ammonia (70:30 v/v)
D. n-butanol-acetic acid-water (74:19:20 v/v)
E. Formic acid-2-butanone-t-butanol-water (15:30:40:15 v/v)
F. i-propanol-pyridine-acetic acid-water (40:40:5:20 v/v)
G. diethyl ether-90% formic acid (7:1 v/v), saturated with water

Amino acids were separated on Silica Gel G plates or on Eastman Chromagram sheets with solvent systems A, B, C or D and detected with 0.2% ninhydrin in 95% ethanol.

Carnosine and histidine were detected with Pauly's diazo reagent (Rockland *et al.*, 1964) on chromatograms developed with solvent A or C. To detect creatine and creatinine the chromatogram, developed in solvent A or C, was sprayed with 1% aq. picric acid solution, heated at 110° for 1 hr, then sprayed with 4% sodium hydroxide solution (Block *et al.*, 1952).

Sugars, phosphates, and purine derivatives were chromatographed on Cellulose MN 300 with solvent E or F. Sugars were revealed with 0.1*M* p-anisidine phthalate in 95% ethanol (Randerath, 1963), and phosphates with Hanes-Isherwood reagent (Bandurski *et al.*, 1951).

Lactic acid was detected with a spray reagent consisting of 0.3% bromphenol blue and 0.1% methyl red in 95% ethanol after chromatography on Cellulose MN 300 developed with solvent E, or on Silica Gel G developed with solvent G. Lactic acid was also detected with Hanes-Isherwood reagent on Silica Gel G (Ting *et al.*, 1965).

Purine derivatives were also separated on DEAE-cellulose with 0.01*N* hydrochloric acid as solvent and detected as dark, absorbing spots under ultraviolet light at 257 mμ (Randerath, 1962). Quaternary ammonium compounds were separated on Silica Gel G with solvent A or C and detected by exposure to iodine vapor (Brante, 1949).

Aroma

Separation of the beef aroma precursors was followed by the development of meaty aroma on pyrolysis. Samples (0.25 ml) in 10 ml beakers were heated on a hot plate (surface temperature 150–160°) until completely dry. The aromas obtained were compared to the aroma produced on pyrolysis of the diffusate in a similar manner.

Four to six members of the Meat Laboratory evaluated the aromas informally. Since only major changes in odor were of interest to us at this time, formal, statistical taste panel procedures were not followed and the subjective, descriptive analyses were used. The panel responses were in agreement in differentiating meaty aromas resembling those of the diffusate control from others also produced on pyrolysis.

RESULTS AND DISCUSSION

OUR CHROMATOGRAPHIC EXPERIMENTS indicate that considerable separation of the low molecular weight components of beef muscle may be achieved by mild treatment. Fractions that gave desirable meat-like aromas on pyrolysis were obtained by separation of the diffusate on Bio-Gel P-2, Amberlite XAD-2, and DEAE-Sephadex, thus excluding a number of compounds from consideration as aroma precursors.

Table 1. Elution sequence of beef diffusate components from a Bio-Gel P-2 column.

1.	Sugar phosphates, peptides, amino acids (most), lactic acid, quaternary amines
2.	Free sugars, creatine, glycine, taurine, serine, urea
3.	Inosinic acid, phenylalanine, inorganic salts
4.	Creatinine
5.	Tyrosine, unknown abs. max. 260 mμ
6.	Inosine
7.	Hypoxanthine
8.	Unknown, abs. max. 265 mμ

The elution sequence of components of the diffusate from Bio-Gel P-2 is shown in Table 1 and Fig. 1.

Most of the amino acids were eluted together, but preliminary experiments indicated that some separation may be achieved within this group. Glycine, taurine and serine were eluted somewhat later followed by phenylalanine. Tyrosine, the last amino acid to be eluted, was well separated from the others. The sugar components were separated into two peaks containing sugar phosphates and free sugars, respectively. The amino acid and sugar peaks essentially overlapped, although the elution of sugars tended to be slightly retarded with respect to the amino acids. Inosinic acid, inosine and hypoxanthine were well separated, emerging from the column in that order.

The order of separation of components on Bio-Gel P-2 indicates that two major mechanisms are in operation: (1) exclusion from the gel on the basis of molecular weight, and (2) adsorption on the surface of the gel. In this respect the principles of separation on P-2 are similar to those on Sephadex (Gelotte, 1960). The elution order of purine derivatives was similar to that observed by Uziel *et al.* (1965) and Schwartz *et al.* (1965) who claimed that the movement of these compounds was governed by adsorption rather than by exclusion (or gel filtration).

Other factors affecting resolution on Bio-Gel P-2, besides column dimensions and flow rate, may be the pH of the eluting medium as well as the presence of other components in the sample. A mixture of six amino acids, carnosine, and sodium chloride was chromatographed using water as eluent.

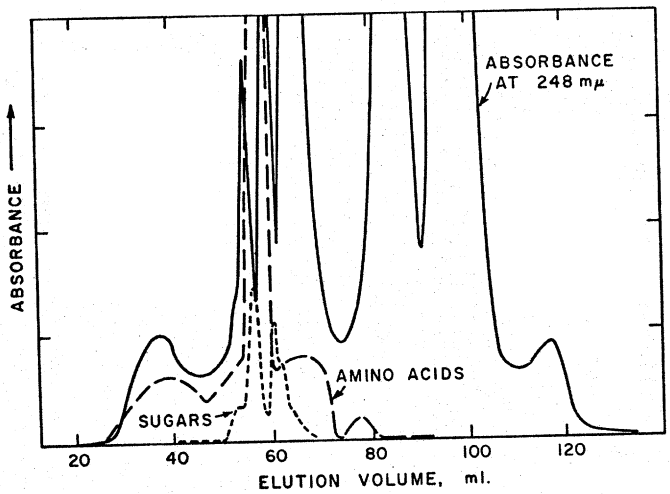

Fig. 1. Beef diffusate chromatographed on Bio-Gel P-2 (100–200 mesh).

Although the individual components were not completely resolved, the following elution sequence was clearly visible: (1) aspartic acid; (2) alanine, valine and leucine; (3) cysteic acid; (4) carnosine; (5) phenylalanine; (6) sodium chloride. However, during chromatography of the diffusate, carnosine was eluted together with alanine, valine and leucine. The presence of acids and bases in the diffusate may have caused this shift in the elution of carnosine. Schwartz *et al.* (1966) in their studies on desalting of amino acids on Bio-Gel P-2 reported that the elution volumes of glutamic acid and arginine were dependent on the pH of the eluting medium.

To obtain sufficient material for organoleptic evaluation, we chromatographed 25 ml of beef diffusate on a 5 × 75 cm column of Bio-Gel P-2 (200–400 mesh). The separation of components was similar to that shown in Fig. 1. The aromas obtained on pyrolysis of the eluted fractions were compared with the aroma of pyrolyzed beef diffusate. The most intense odors were obtained from fractions containing both amino acids and sugars. The composition of the fractions corresponding to the two sugar peaks (Fig. 1) are shown in Table 2.

The first fraction, containing glucose-6-phosphate, yielded a meaty aroma very similar to that of the diffusate. The odor from the second fraction, containing the free sugars—glucose, fructose and ribose—was also meat-like but had a pronounced plant-like or grassy character. Fraction 1, which yielded the most meat-like aroma thus could be regarded as containing aroma precursor compounds.

Among the components identified in this fraction are a number of amino acids, carnosine, anserine, phosphate sugars, quaternary amines and lactic acid. Trace amounts of creatine and inorganic salts were also found in this fraction, although the bulk of these compounds was found

Table 2. Composition of flavor fractions of beef diffusate separated on Bio-Gel P-2.

Fraction 1		Fraction 2
Aroma—meaty		Aroma—meat-like, grassy
1-methyl-histidine	Cysteic acid[1]	Taurine
Lysine	Taurine[1]	Urea
Histidine	Serine[1]	Aspartic acid
Anserine	Asparagine[1]	Glutamic acid
Carnosine	Glutamine[1]	Serine
Threonine	Arginine[1]	Threonine
Proline	Glucose 6-phosphate	Asparagine
Glutamic acid	Unknown sugar phosphate	Glutamine
Glycine	Lactic acid	Glycine
Alanine	Creatine[1]	Methionine
Valine	Choline	Phenylalanine
Methionine	Carnitine	Alanine[1]
Isoleucine	Organic phosphates	Carnosine[1]
Leucine	$PO_4\equiv$[1]	Inosinic acid
		Creatine
		Creatinine
		$PO_4\equiv$
		Organic phosphates
		Glucose
		Fructose
		Ribose

[1] Trace amount.

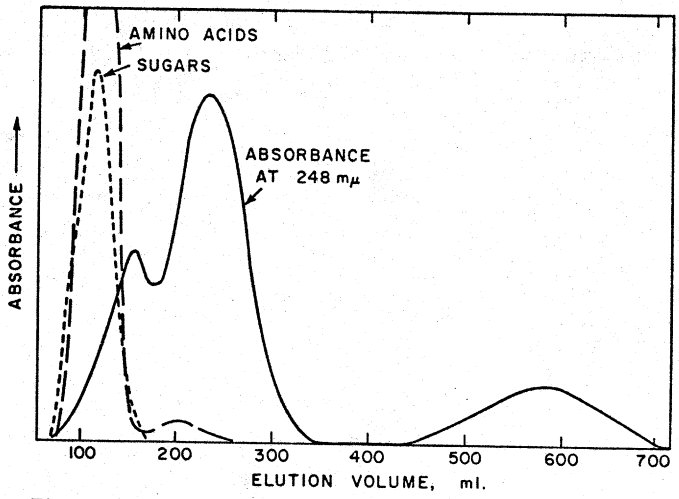

Fig. 2. Beef diffusate chromatographed on Amberlite XAD-2 (20–50 mesh).

in other fractions. The odor precursor fraction was free from purines and their derivatives as well as from other aromatic compounds.

Another type of separation of the components of beef diffusate was afforded by chromatography on crosslinked polystyrene polymer, Amberlite XAD-2. Three ultraviolet absorbing peaks were obtained when the diffusate was separated on an XAD-2 column (Fig. 2). The bulk of the material, listed in Fraction 1, Table 3, was located in the first UV-absorbing peak. This fraction contained most of the amino acids, all sugars, inosinic acid, and other polar compounds.

The subsequent fractions contained compounds which possess a high degree of aromaticity or nonpolar groups. The presence of a large hydrocarbon chain in leucine and isoleucine or of an aromatic ring in phenylalanine and tyrosine resulted in the retardation of these compounds on XAD-2 resin, making the separation of these amino acids possible.

The order of elution of hypoxanthine and inosine from the XAD-2 resin was the reverse of that observed from the P-2 gel. This may be due to the decreased ionic character of inosine as a result of substituting the sugar moiety for the N-9 hydrogen of the purine nucleus.

Table 3. Composition of fractions of beef diffusate separated on Amberlite XAD-2.

Fraction 1	Fraction 2	Fraction 3
Aroma: meaty	Aroma: pungent, grassy	Aroma: none
Amino acids (all except those in Fraction 2)	Leucine	Inosine
Sugars	Isoleucine	
Phosphates	Phenylalanine	
Lactic acid	Tyrosine	
Creatine	Hypoxanthine	
Creatinine	Unknown, abs. max. 265 mμ	
Inosinic acid	Unknown, abs. max. 283 mμ	
Inorganic salts		

On pyrolysis Fraction 1 gave a meaty aroma, similar to that of the diffusate. The odor of pyrolyzed Fraction 2 was pungent and plant-like; Fraction 3 gave little or no aroma.

Since the meaty fractions from the separations on P-2 gel and XAD-2 resin contained sugar phosphates or inosinic acid and these compounds were implicated in flavor development (Wood, 1961; Batzer *et al.*, 1962), it was of interest to observe the effect of the removal of these anions on aroma. The diffusate was washed through a column of DEAE-Sephadex [$HCOO^-$] with 0.1*N* formic acid. The total material eluted contained no phosphorus and gave on pyrolysis a meaty aroma quite similar to that of the diffusate.

In subsequent separations of the diffusate on DEAE-Sephadex, fractions of the eluate were collected. The absorbance of the eluted fractions monitored at 248 mμ is shown in Fig. 3. Of the four peaks obtained, the first had an absorption maximum at 260 mμ; the second peak, with a maximum at 247 mμ, contained hypoxanthine and inosine. Peaks 3 and 4 showed absorption maxima at 265 mμ and 283 mμ respectively. The compounds responsible for the ultraviolet absorption in peaks 1, 3 and 4 are at present unidentified. (The spectra referred to were measured in 0.1*N* formic acid.)

The amino acids were eluted from DEAE-Sephadex in the order expected from their isoelectric points (Cohn *et al.*, 1943). They were distributed into five groups: basic, neutral and phenylalanine, tyrosine, taurine, and glutamic acid in the order of their elution. On the basis of these separations, and the analysis of other components, the effluent was divided into seven fractions as shown in Table 4.

Factors other than ion exchange also influence the separation of compounds on DEAE-Sephadex. Adsorption and molecular sieving effects most likely govern the elution of aromatic and non-ionic substances. The retardation of purine derivatives and tyrosine is probably due to adsorption effects. Free sugars are considerably retarded on DEAE-Sephadex emerging together with tyrosine, probably as a result of gel permeation effects. Öckerman

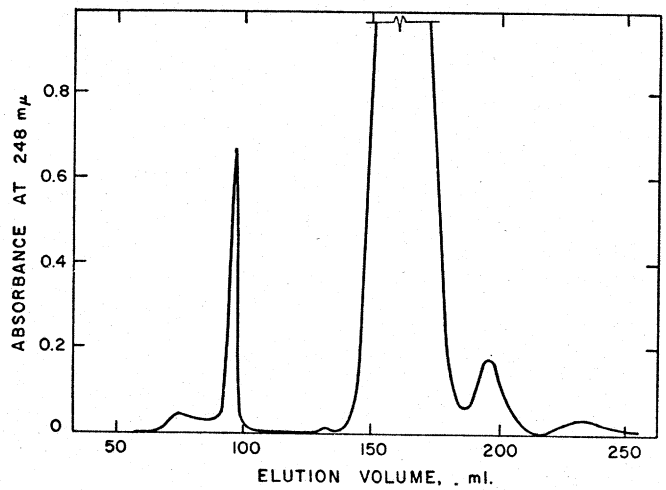

Fig. 3. Beef diffusate chromatographed on DEAE-Sephadex A-25 [$HCOO^-$].

Table 4. Composition of fractions of beef diffusate eluted from DEAE-Sephadex [$HCOO^-$] with 0.1*N* formic acid.

Fraction number						
1	2	3	4	5	6	7
Aroma		Aroma	Aroma			
Carnosine	Creatine	Threonine	Tyrosine	Taurine	Glutamic acid	Glutamic acid
Anserine	Creatinine	Serine	Glucose	Inosine	Unknown, abs. max. 265 mμ	Unknown, abs. max. 283 mμ
Lysine		Asparagine	Fructose	Hypoxanthine		
1-methyl-histidine		Glutamine	Ribose			
Histidine		Proline	Inosine			
Arginine		Glycine	Hypoxanthine			
Ammonia		Alanine				
Creatinine		Valine				
Choline		Methionine				
Carnitine		Isoleucine				
Unknown, abs. max. 260 mμ		Leucine				
		Phenyl-alanine				
		Creatine				

(1963) reported that glucose emerges after glycogen (polysaccharide) from DEAE-Sephadex and attributes this separation to residual molecular sieving properties of unsubstituted Sephadex.

Three of the fractions yielded odors on pyrolysis. However, these odors did not closely resemble the aroma from the diffusate. Fraction 1, containing the basic compounds, yielded an amine-like odor reminiscent of lamb. The odor from the neutral amino acid Fraction 3 was pungent and potato-like. The sugar-containing Fraction 4 produced a fainter sweet, acrid odor. Combination of Fraction 4 with either Fraction 1 or 3 resulted in more meat-like aromas, while combination of Fractions 1, 3 and 4 resulted in an aroma very similar to that of beef diffusate. Apparently the components in Fractions 2, 5, 6 and 7 do not contribute significantly to the meat aroma.

CONCLUSION

THE SEPARATIONS OF BEEF DIFFUSATE on Bio-Gel P-2, Amberlite XAD-2 and DEAE-Sephadex yielded fractions that gave good meaty aromas on pyrolysis, each containing variations in the constituent components. While we still have not identified the precursors of the characteristic meaty aroma, it is possible to eliminate a number of meat components that do not play an important role in flavor development. Components separated from meat aroma-producing fractions were considered not to contribute significantly to the basic meaty aroma. However, these compounds could contribute to the overall odor of broiled or roasted meat.

Although amino acids and sugars are present in all flavor fractions, there do not appear to be any requirements for specific components. In the flavor fraction from P-2 gel only sugar phosphates were present, while the free sugars were equally effective in producing a meaty aroma in the DEAE-Sephadex fractions.

The separation of the amino acids in the flavor fractions is not sufficiently clear at present to evaluate their individual contributions to the meat flavor; but tyrosine, phenylalanine, taurine and glutamic acid may be removed without seriously affecting the aroma. Creatine and creatinine may not be involved in aroma development; they can be removed almost entirely from the flavor fractions by passage through the Bio-Gel P-2 or DEAE-Sephadex.

Purines and purine derivatives have been implicated in the development of meat flavor and aroma (Batzer *et al.*, 1962); however, we excluded inosinic acid, inosine and hypoxanthine from the aroma fraction by chromatography on Bio-Gel P-2. Hypoxanthine and inosine also can be removed by separation on XAD-2 while treatment with DEAE-Sephadex removes inosinic acid. It appears, therefore, that these purines are not involved in the development of meaty aroma.

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